of the 5'-Flap and F_{adj} strands. The 5'-Flap and F_{adj} strands are hybridized immediately contiguously with one another to the same F_{br} strand.

FIG. 9B. Nucleotide sequence of 5'-Flap Substrate 4 (AI4/HJ46/HJ73; SEQ ID NO:64/SEQ ID NO:65/SEQ ID NO:66), which contains a one nucleotide gap at the junction of the 5'-Flap and F_{adj} strands.

FIG. 9C. Nucleotide sequence of 5'-Flap Substrate 5 (AI4/HJ46/HJ74; SEQ ID NO:64/SEQ ID NO:65/SEQ ID NO:67), which contains a 3 nucleotide gap at the junction of the 5'-Flap and F_{adj} strands.

FIG. 9D. Nucleotide sequence of 5'-Flap Substrate 6 (AI4/HJ46/HJ75; SEQ ID NO:64/SEQ ID NO:65/SEQ ID NO:68), which contains a 5 nucleotide gap at the junction of the 5'-Flap and F_{adj} strands.

FIG. 10. Nucleotide sequence of Pseudo Y-Structure (AI4/HJ46; SEQ ID NO:64/SEQ ID NO:65).

FIG. 11. Nucleotide sequence of Double Flap Substrate 1 (AI4/HJ46/HJ77; SEQ ID NO:64/SEQ ID NO:65/SEQ ID NO:69), which contains a one nucleotide 3'-flap. The 5'-Flap and F_{adj} (3'-Flap) strands are hybridized immediately contiguously with one another to the same F_{br} molecule.

FIG. 12. Nucleotide sequence of Double Flap Substrate 2 (AI4/HJ46/HJ78; SEQ ID NO:64/SEQ ID NO:65/SEQ ID NO:70), which contains a ten nucleotide 3'-flap. The 5'-Flap and F_{adj} (3'-Flap) strands are hybridized immediately contiguously with one another to the same F_{br} molecule.

FIG. 13. Effect of FEN-1 Concentration in the Mobility Shift Assay. Various amounts of purified FEN-1 were incubated with 10 fmol of labeled 5'-Flap Substrate 2 (FIG. 8) as described in Example 4, "*Materials and Methods.*" *Lanes 1-5*, 0, 10 (0.2 pmol), 20 (0.4 pmol), 40 (0.8 pmol), and 80 (1.6 pmol) ng of purified FEN-1, respectively. The structure of the labeled binding 5'-Flap Substrate 2 (FIG. 8) is shown at the top. Individual strands are illustrated as solid lines with a half-arrow at the 3'-end and are designated F_{adj} strand *(top)*, flap strand *(bottom left)*, and F_{adj} strand *(bottom right)*. The position of the radioactive end label is indicated by an *asterisk*. On the right, the positions of free and complexed labeled 5'-Flap Substrate 2 are shown. The gradual shift in the free probe is due to the binding of

labeled 5'-Flap Substrate 2 by FEN-1, followed by release during electrophoresis, which is more apparent with increasing FEN-1 concentration.

FIG. 14. Effect of Nonspecific Nucleic Acid Competitors on FEN-1 Binding. Binding reactions were carried out as described in Example 4, "*Materials and Methods.*" Each reaction contained 60 ng(1.2 pmol) of purified FEN-1 and the indicated amounts of the following nucleic acid competitors: *lanes 1* and *2*, no competitor; *lanes 3-6*, AI4/CLH6 (SEQ ID NO:64/SEQ ID NO:73); *lanes 7-10*, AI4; *lanes 11-14*, tRNA. The competitor structures and concentrations are indicated below their respective lanes. When more than one oligonucleotide composes a structure *(e.g.* AI4/CLH6 in a double-stranded configuration) in this or any of the figures that follow, the mass given is only for the oligonucleotide AI4 (SEQ ID NO:64), and an equimolar quantity of the other oligonucleotides listed in the name of the structure is added in addition to constitute the complete competitor structure. The structure of the labeled 5'-Flap Substrate 2 (FIG. 8) is illustrated at the top (see the legend to FIG. 13 for details). The positions of free and complexed labeled substrates are indicated on the right.

FIG. 15 Competition of FEN-1 Binding Activity with DNA Flap Substrate Derivatives. Binding reactions were carried out as described in Example 4, "*Materials and Methods*" and contained 60 ng (1.2 pmol) of purified FEN-1. In addition, the indicated amounts of the following nucleic acid competitors were present: *Lanes 1* and *2*, no competitor; *lanes 3-6*, 5'-Flap Substrate 3 (FIG. 9A); *lanes 7-10*, Pseudo Y-Structure (FIG. 10); and *lanes 11-14*, AI4/HJ47 (SEQ ID NO:64/SEQ ID NO:58). The structures of the labeled 5'-Flap Substrate 2 (FIG. 8) and the unlabeled competitors are illustrated above and below the figure, respectively. Structure designations are as described in the legend to FIG. 13. On the right, the positions of free and complexed substrates are shown.

FIG. 16 Competition with 5'-Flap Substrates Containing Recessed $F_{adj.}$ Strands. Binding reactions were carried out as described in Example 4, "*Materials and Methods*" and contained 40 ng (0.8 pmol) of purified FEN-1. In addition, the indicated amounts of the following nucleic acid competitors were present: *lanes 1* and *2*, no competitor; *lanes 3-7*, 5'-Flap Substrate 3 (FIG. 9); *lanes 8-12*, 5'-Flap Substrate 4 (FIG. 9B); and *Lanes 13-17*, 5'-Flap Substrate 5 (FIG. 9C); *lanes 18-22*, 5'-Flap Substrate 6 (FIG. 9D); and *lanes 23-27*, Pseudo Y-Structure (FIG. 10). Note that the 5'-Flap Substrate 3 (FIG. 9A) competitor was

titrated from 2 to 32 ng, while all other competitors were titrated from 4 to 64 ng. The structures of the labeled 5'-Flap Substrate 2 (FIG. 8) and the unlabeled competitors are illustrated above and below the figure, respectively. Structure designations are as described in the legend to FIG. 13. On the right, the positions of free and complexed substrates are shown. *nt*, nucleotide.

FIG. 17. Demonstration that 3'-Flap Strands Can Serve as an F_{adj} Strand. Panel A: Gel mobility shift assay demonstrating that FEN-1 endonuclease binds double flap structures. 5'-Flap Substrate 2 (FIG. 8, 10 fmol, 5'-end labeled at the 5'-Flap strand) was incubated in 20 μl buffer (50mM Tris, pH8, 20 mM NaCl, 5mM EDTA, 10% glycerol, 50 μg BSA) with 40 ng (0.8 pmol) purified FEN-1 for 5 min. at 20°C. In addition, the indicated amounts of the following nucleic acid competitors were present: *lanes 1 and 2*, no competitor; *lanes 3-6*, 5'-Flap Substrate 3 (FIG. 9A); *lanes 7-10*, Pseudo Y-Structure (FIG. 10); *lanes 11-14*, Double Flap Substrate 1 (FIG. 11); and *lanes 15-18*, Double Flap Substrate 2 (FIG. 12). Reaction products were separated on a native 5% polyacrylamide gel and the bands were visualized and quantified by exposure to PhosphorImager screens. The positions of the free and complexed substrates are shown on the right. The masses indicated for the competitors structures is only for the F_{br} strand (AI4; SEQ ID NO:64). Panel B: Demonstrates the ability of FEN-1 endonuclease to cleave the 5'-Flap strand of Double Flap Substrates. Various amounts of FEN-1 were incubated with 5'-end labeled 5'-Flap Substrate 2 (FIG. 8), 5'-end labeled Pseudo Y-Structure (FIG. 10), 5'-end labeled Double Flap Substrate 1 (FIG. 11) and 5'-end labeled Double Flap Substrate 2 (FIG. 12) as described above. *Lanes 1, 6, 11 and 16*, 0 units of FEN-1; *lanes 2, 7, 12 and 17*, 3 units of FEN-1; *lanes 3, 8, 13 and 18*, 1 unit of FEN-1; *lanes 4, 9 14 and 19*, 0.33 units of FEN-1; *lanes 5, 10, 15 and 20*, 0.11 units of FEN-1. Reaction products were separated on a 10% denaturing polyacrylamide gel and visualized by autoradiography. The locations of substrate and product bands (in nucleotides) are indicated on the right.

FIG. 18 Important Substrate Elements Required for Efficient Binding to Nicked Duplex DNA. Forty nanograms of purified FEN-1 (0.8 pmol) was incubated with 0.2 ng (8 fmol) of labeled nicked duplex DNA (AI4/CLH2/CLH3; SEQ ID NO:64/SEQ ID NO:71/SEQ ID NO:72) and the indicated amounts of each unlabeled competitor. *Lanes 1* and *2*, no competitor *lanes 3-6*, AI4/CLH2/CLH3 (SEQ ID NO:64/SEQ ID NO:71/SEQ ID

NO:72); *lanes 7-10,* AI4/CLH6 (SEQ ID NO:64/SEQ ID NO:73); *Lanes 11-14,* AI4/CLH2 (SEQ ID NO:64/SEQ ID NO:71); *lanes 15-18,* AI4/CLH3 (SEQ ID NO:64/SEQ ID NO:72). The structures of the substrate and competitors are shown schematically with designations as described in the legend to FIG. 13.--

At Col. 56, after line 65 and before line 66, please insert the following text:

--EXAMPLE 4 DNA Structural Elements Required for FEN-1 Binding

This Example demonstrates the ability of FEN-1 endonuclease to bind and cleave varius 5'-flap structures and 3',5'-double flap structures.

Materials and Methods

Purification of FEN-1. FEN-1 was overexpressed in *E. coli* and purified as described previously (*see* Harrington and Lieber, 1994, *Genes & Dev.* 8:1344-1355). The final purity of FEN-1 was >95%.

Oligonucleotides. All DNA oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). The sequence of each is shown after its name and is written 5' to 3':

SC1: CAGCAACGCAAGCTTG (SEQ ID NO:59);

SC3: GTCGACCTGCAGCCCAAGCTTGCGTITGCTG (SEQ ID NO:61);

SC5: ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC (SEQ ID NO:63);

AI4: GGCCGTATCTGGGTFCGAATTCATCAAGGGACATCTCCTAC (SEQ ID NO:64);

HJ46: CCTATCGTTGCATGGTCCCAGAACCCAGATACGGC (SEQ ID NO:65);

HJ47: GTAGGAGATGTCCCTFGATGAATTC (SEQ ID NO:58);

HJ73: GTAGGAGATGTCCCTTGATGAATT (SEQ ID NO:66);

HJ74: GTAGGAGATGTCCCTTGATGATTC (SEQ ID NO:67);

HJ75: GTAGGAGATGTCCCTTGATG (SEQ ID NO:68);

HJ77: GTAGGAGAT GTCCCTTGATGAATTCC (SEQ ID NO:69);

HJ78: GTAGGAGATGTCCCTTGATGAATTCCTTGTGTTTC (SEQ ID NO:70);

CLH2: GTAGGAGATGTCCCTI'GATGAATF (SEQ ID NO:71);

CLH3: CGAACCCAGATACGGC (SEQ ID NO:72); and

CLH6: GTAGGAGATGTCCCTTGATGAATTCGAACCCAGATACGGC (SEQ ID NO:73).

Flap Endonuclease Assay. The standard endonuclease assay has been described previously (*see* Harrington and Lieber, 1994, *EMBO J.* 13:1235-1246). Briefly, FEN-1 endonuclease activity was measured in a 15-μl reaction containing 50 mM Tris (pH 8), 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin, and 10 fmol of labeled substrate. Reactions were carried out at 30°C for 30 min and terminated by adding 15 μl of 95% formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, 1 mg/ml xylene cyanol. The tubes were heated to 95°C for 5 min, loaded onto a 10% polyacrylamide gel containing 7 M urea and 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8)), and run for 90 min at 75 watts. One unit of FEN-1 activity produces 1 fmol of cleaved product under these standard reaction conditions.

Mobility Shift Assay for FEN-1. Binding reactions were carried out in a 20 μl reaction containing 50 mM Tris (pH 8), 10 mM NaCl, 5 mM EDTA, 10% glycerol, 50 μg/ml bovine serum albumin, 0.25 ng (10 fmol) of labeled oligonucleotide probe, and the indicated amount of FEN-1. Following a 5-min incubation at 20°C, the reactions were loaded onto a 5% polyacrylamide gel containing 0.5 X TBE and run at 125 V for 90 mm. The gels were dried and exposed to PhosphorImager screens to allow quantitation of bands. When more than one oligonucleotide composes a multi-oligonucleotide competitor structure (*e.g.* AI4/HJ46/HJ47 in 5'-Flap Substrate 3; FIG. 9A) in any of FIGS. 13-18, the mass given in the figure is only for the oligonucleotide AI4 (SEQ ID NO:64), and an equimolar quantity of the other oligonucleotides listed in the name of the structure is added in addition to constitute the complete competitor structure.

Results

The ability of FEN-1 to bind 5'-flap substrates was demonstrated in the mobility shift assay described above. Because FEN-1 is a potent nuclease in the presence of divalent metal ions, EDTA was included in the binding reaction to remove residual metal ions. Under these conditions, FEN-1 was found to bind to the DNA 5'-Flap Substrate 2 (FIG. 8) in a dose-dependent fashion (FIG. 13). This dose response was linear between 10 and 60 ng of FEN-1 under the standard binding conditions. In this range of FEN-1 concentrations, a single shifted species was observed. Although we cannot rule out Mg^{2+} being involved in the binding, we believe this to be unlikely because the substrate binding properties of purified FEN-1 in the absence of Mg^{2+} are entirely consistent with the endonucleolytic activity of FEN-1 in the presence of Mg^{2+} (see below).

Cont a2

To determine the specificity of FEN-1 for the 5'-Flap Substrate 2 (FIG. 8) in the mobility shift assay, we titrated various nucleic acid competitors into the binding reaction (FIG. 14). Double- and single-stranded DNAs compete inefficiently in this assay. An ~1000-fold molar excess of these competitors was needed to fully compete the binding of FEN-1 away from the labeled 5'-Flap Substrate 2 (FIG. 8). tRNA failed to compete for FEN-1 binding even when present in a 1000-fold molar excess over labeled 5'-Flap Substrate 2 (FIG. 8). These results are consistent with the inhibition profiles of these nucleic acid competitors in the standard flap endonuclease assay (*see* Harrington and Lieber, 1994, *EMBO J.* 13:1235-1246).

The binding specificity of FEN-1 was further characterized by titrating derivatives of the 5'-Flap Substrate 2 (FIG. 8) into the reaction (FIG.15). Unlike double- and single-stranded DNAs, a 40-fold excess of unlabeled 5'-Flap Substrate 3 (FIG. 9A) was sufficient to fully compete the binding of FEN-1 away from the labeled 5'-Flap Substrate 2 (FIG. 8). In addition, we found that the entire flap structure was required for optimal competition to occur. The Pseudo Y-Structure (FIG.10), which was missing the F_{adj} strand, competed 3-fold less efficiently than the 5'-Flap Substrate 3 (FIG. 9A). Furthermore, the 5'-overhang, which was missing the flap strand (see FIG. 15), competed 4-fold less efficiently than the 5'-Flap Substrate 3 (FIG. 9A), but 5-fold more efficiently than single-stranded DNA. This indicates that the presence of the F_{adj} strand enhances the binding of FEN-1 to this overhang structure. Thus, the reduction in binding of FEN-1 to the Pseudo Y-Structure (FIG.10) (compared with

the 5'-Flap Substrate 3 (FIG. 9A)) and the enhancement in binding of FEN-1 to the 5'-overhang (compared with single-stranded DNA) demonstrate the importance of the F_{adj} strand in the binding step of this reaction

In addition to binding, we have shown that the F_{adj} strand is required for efficient cleavage of the flap strand. The efficiency of cleavage correlated with the proximity of the 3'-end of the F_{adj} strand to the elbow of the flap strand (*see* Harrington and Lieber, 1994, *EMBO J.* 13:1235-1246). We were interested in determining whether these recessed F_{adj} strands were cleaved inefficiently due to a reduced ability of FEN-1 to bind to these substrates. To test this, we carried out the binding reaction in the presence of unlabeled 5'-flap structures with various gap sizes between the F_{adj} strand and the flap strand elbow (FIG. 16). We found that the ability of FEN-1 to bind to the flap structure efficiently was dependent upon the proximity of the F_{adj} strand to the elbow of the flap strand. This result suggests that the inability of FEN-1 to efficiently cleave 5'-flap structures with large gaps between the F_{adj} strand and the flap strand elbow is due, at least in part, to the failure of FEN-1 to bind to this substrate. Interestingly, FEN-1 binds to a 5-nucleotide gap (5'-Flap Substrate 6; FIG. 9D) more efficiently than to a Pseudo Y-Structure. This indicates that the 5-nucleotide recessed F_{adj} strand is capable of partially stabilizing FEN-1 binding, thereby suggesting that FEN-1 contacts the F_{adj} strand at >5 nucleotides from the flap strand elbow.

Cont a2

We have demonstrated the importance of the F_{adj} strand for the binding and cleavage of a 5'-flap structure by FEN-1. The exact role that the F_{adj} strand plays in the recognition of this structure by FEN-1, however, is not clear. For example, does FEN-1 require a fully base-paired 3'-terminus located in juxtaposition to the elbow of the flap strand, or does FEN-1 simply require that the region near the elbow of the flap strand be double-stranded? To determine which of these two possibilities is correct, we designed a double flap substrate that contained an F_{adj} strand that had either 1 nucleotide (Double Flap Substrate 1; FIG. 11) or 10 nucleotides (Double Flap Substrate 2; FIG. 12) of extra sequence at the 3'-end. Thus, the base that contained the 3'-terminus was neither base-paired nor located in immediate juxtaposition to the elbow of the flap strand. This substrate, called a double flap structure, contained both a 5'- and a 3'-flap strand. Previously, we have shown that FEN-1 is not capable of cleaving 3'-flap strands (*see* Harrington and Lieber, 1994, *EMBO J.* 13:1235-1246). Here, we were interested in determining whether a displaced 3'-flap strand can serve

as an F_{adj} strand to allow efficient binding to this structure by FEN-1. In the mobility shift assay, we found that FEN-1 could bind a double flap efficiently (FIG. 17A). Double Flap Substrate 1 (FIG. 11) and the 5'-Flap Substrate 3 (FIG. 9A) competed 3-5-fold more efficiently than Double Flap Substrate 2 (FIG. 12), which, in turn, competed 2-fold more efficiently compared with the Pseudo Y-Structure (FIG. 10). This indicates that both a 1-nucleotide and a 10-nucleotide 3'-flap strand can serve as an F_{adj} strand in the binding step of this reaction. Furthermore, this result indicates that the F_{adj} strand is required to create a double-stranded region next to the elbow of the flap strand rather than to supply a base-paired 3'-terminus.

The ability of FEN-1 to bind to a double flap structure suggests that FEN-1 may be capable of cleaving the 5'-flap strand on this structure. To test this, we carried out the standard flap endonuclease assay. As determined previously (*see* Harrington and Lieber, 1994, *EMBO J.* 13:1235-1246), FEN-1 cleaves the flap strand of a 5'-flap structure efficiently; however, it cleaves a pseudo Y-structure ~100-fold less efficiently. When Double Flap Substrates 1 and 2 (FIGS. 11 and 12, respectively) were tested in this cleavage assay, we found that the 1- and 10-nucleotide 3'-flap strands served as an F_{adj} strand, allowing FEN-1 to cleave the 5'-flap strand efficiently (FIG. 17B). Furthermore, cleavage of the Double Flap Substrates 1 and 2 (FIGS. 11 and 12, respectively) by FEN-1 was more efficient than that of the standard 5'-Flap Substrate 3 (FIG. 9A).

In addition to flap cleavage activity, FEN-1 has been shown to have a 5'-3'-exonuclease activity that is dependent upon double-stranded DNA. Like the flap endonuclease, the 5'-3'-exonuclease is stimulated by the presence of an F_{adj} strand. Thus, the FEN-1 exonuclease is optimally active at a nick. To test the relative binding efficiency of FEN-1 on 5'-flap structures *versus* nicks, we compared the ability of these structures to compete in the mobility shift assay using a labeled flap substrate. We found that in this assay, a 5'-flap structure competed 2-4 times more efficiently than a nick structure (data not shown). In the cleavage assay, we found that FEN-1 cleaved flap structures severalfold more efficiently than nicks; however, there appears to be considerable variation among nicked DNA substrates. Substrate preference has been observed previously with CCA exonuclease (*see* Goulian *et al.*, 1990, *J. Biol. Chem.* 265:18461-18471), an enzyme that we believe to be

the same as FEN-1. The basis for variability in substrate preference is not known at this time.

The ability of nicked DNA to compete with FEN-1 binding to a 5'-flap structure suggests that we could use the mobility shift assay to dissect the important substrate features necessary for FEN-1 binding to DNA nicks. To test this, we labeled a nicked DNA substrate and carried out the mobility shift assay under standard binding and electrophoresis conditions. In this assay, FEN-1 did bind to a labeled nicked DNA substrate and retard its mobility on a native gel (FIG. 18; *lanes 1* and *2*). Upon addition of unlabeled nicked DNA, the shifted substrate was no longer detectable in the presence of an 80-fold molar excess of competitor. In contrast, titration of unlabeled double-stranded DNA lacking a nick but containing the same nucleotide sequence failed to compete FEN-1 away from the labeled substrate completely, even in the presence of a 250-fold molar excess. This indicates that FEN-1 is recognizing some component of the nicked DNA structure. Analysis of the individual contribution of the F_{adj} strand and the 5'-recessed strand demonstrates that the F_{adj} strand is important in the binding step of this reaction. This conclusion is consistent with the observation that the calf thymus 5'-3'-exonuclease, the bovine analog of mouse FEN-1, requires the F_{adj} strand for efficient exonuclease activity (*see* Murante *et al.*, 1994, *J. Biol Chem.* 269:1191-1196).--

IN THE CLAIMS

Please add the following new Claims 7-73:

--7. A method of cleaving a polynucleotide, comprising the steps of:

(a) contacting a sample suspected of containing a target nucleic acid of interest, said target nucleic acid comprising a first portion and a second portion located immediately 3' to the first portion, with:

(i) a 5'-polynucleotide probe comprising a 3'-region that is capable of specifically hybridizing to the first portion of the target nucleic acid and a 5'-region located immediately 5' to the 3'-region; and